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An image analysis method for determination of spatial colonization patterns of bacteria in plant rhizosphere

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Abstract A method that allows the rapid visualization of bacterial spatial colonization patterns on roots for the determination of general colonization trends was developed. This method, which analyzes images of roots, and bioluminescence-enhanced images of bacterial colonization patterns on these roots, was used to study the colonization patterns of seed-applied *Enterobacter cloacae* strain E6 on 3-day-old cucumber plants. Conventional dilution-plating methods indicated that E6 colonized cucumber tap roots in high populations and that these populations significantly decreased as the distance from the seed increased. In addition to confirming these observations, image analysis indicated that colonization by E6 significantly decreased on lateral roots as the distance increased horizontally away from the tap root, and that this bacterium did not evenly cover the most densely colonized regions of the cucumber root system. Results from these experiments indicate that the majority of E6 populations on cucumber roots after seed application are limited to the upper regions of the tap root and that E6 does not effectively colonize other regions of the root system.

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Introduction

It is well established that microbial colonization of subterranean plant parts is associated with beneficial activities such as plant disease control, plant growth promotion, and bioremediation (Davison 1988; Weller 1988; Anderson et al. 1993). Unfortunately our understanding of colonization processes is limited due to inadequate methodologies and the complex nature of studies of root systems in soil (Stanghellini and Rasmussen 1989; Handelsman and Staab 1996). Colonization of roots by plant-beneficial and other plant-associated bacteria is most frequently analyzed using conventional, labor-intensive dilution-plating techniques (Kloepper and Beauchamp 1992). These methods estimate mean population sizes of bacteria but do not yield complete information regarding their distribution throughout the rhizosphere without considerable effort (Bahme and Schroth 1987). The lack of information regarding the spatial distribution of bacteria in the rhizosphere warrants the development of methods that rapidly provide representative information regarding the spatial distribution of beneficial bacteria throughout the rhizosphere (Stanghellini and Rasmussen 1989). In this study, we describe a method that utilized image processing to determine spatial colonization patterns of seed-applied *Enterobacter cloacae* E6 on roots of 3-day-old cucumber plants. A preliminary description of this method has been reported (Roberts et al. 1994).

Materials and methods

Bacterial strains and seed bacterization

All bacteria were grown in Luria-Bertani (LB) medium (Miller 1972) unless otherwise noted. When appropriate, media were supplemented with the antibiotics kanamycin (Km) at 50 μg/ml, rifampicin (Rif) at 100 μg/ml, or cycloheximide at 100 μg/ml. The bacterial *lux* gene cassette on plasmid pUCD607 (Shaw and Kado 1986) was previously mobilized into *E. cloacae* E6

(ATCC39978), which resulted in a bioluminescent strain (Fravel et al. 1990). A spontaneous, Rif-resistant mutant of *E. cloacae* E6(pUCD607) was isolated by standard methods (Miller 1972). This Rif-resistant mutant, designated E6R6(pUCD607), was similar in growth rate and colony morphology to the parent strain. *E. cloacae* strains were grown and applied to cucumber seeds (*Cucumis sativum* L. cv Markertmore 76) essentially as described (Roberts et al. 1992). Bacterization of seeds resulted in approximately 10^9 cfu per seed.

Analysis of populations of *E. cloacae* along cucumber taproots

Seeds bacterized with E6R6(pUCD607) were sown 2 cm below the soil surface in cylinders (9 cm × 3 cm diameter) filled with nonsterile Galestown gravelly loamy sand field soil (77.8% sand, 12.6% silt, 9.6% clay, 0.6% organic matter, pH 5.8). Each cylinder was inserted into a 12-cm-high soil column to position sown seeds 7.5 cm above the top of the soil surface in the column. Soil columns were partially submerged in water so that the water level was maintained approximately 12 cm below the surface of the soil column. All soil columns were incubated in a growth chamber at 25 °C with a 12-h day/night illumination cycle. After 72 h, cucumber taproots were segmented into 1-cm pieces beginning immediately below the seed, suspended in sterile distilled water, sonicated for 5 min, and dilution-plated.

Growth of plants in root boxes

Root boxes were constructed from Nalgene carboy lids (105 mm diameter × 20 mm depth) (see Fig. 1A) by cutting a 20-mm slot into one side of the lid lip and cutting a flat edge (75 mm width) on the opposite side of the lid. Five bolts were inserted through the lid around the perimeter and secured with epoxy resin so that the end of the bolts protruded ca. 5 mm above the lip of the carboy lid. Each root box was filled with the same nonsterile field soil described above and covered with a Plexiglas plate that had holes drilled to accommodate the bolts. Root boxes were placed with the flat-edge down into a soil column with the Plexiglas plate at a 60° angle relative to the soil surface, to allow the developing root system to grow along the Plexiglas as previously described (Fravel et al. 1990). Root boxes were equilibrated on the soil column for at least 16 h and subsequently sown with an individual bacterized seed placed 2 cm below the soil surface. This positioned the seeds 7.5 cm above the surface of the soil column. Root boxes were incubated in a growth chamber at 25 °C with a 12-h day/night illumination cycle for the duration of the experiment.

Photography and image digitization

Root boxes were disassembled after 72 h by removing the Plexiglas plate. The exposed cucumber root system and surrounding soil were illuminated at 170 microeinsteins/m^2s and photographed with a Nikkor 60 mm (1 = 2.8) microlens at an exposure time of 1/250 s using Kodak TMax 3200 high-contrast black and white film (Eastman Kodak Co., Rochester, NY) (Fig. 1A). Photographic images of colonization patterns of the bioluminescent strain E6(pUCD607) were generated from agar press plates of the plant root and soil system (Fravel et al. 1990). Press plates were constructed by completely filling 14-cm-diameter Petri dishes to the top of the lid with LB agar (3%) supplemented with Km and cycloheximide. The agar plate was firmly pressed onto the surface of the disassembled root box to imprint the exposed cucumber root structure. Plates were tapped lightly to remove any adhering soil, and incubated for 16 h at 20 °C. Size and orientation of press plates were referenced by marking imprints of bolts on the agar using luminescent paint (Palmer Paint Products, Inc., Troy, Mich.), and placing a 1-cm strip of luminescent tape on the agar surface. Press plates were photographed in absolute darkness using the camera and film described above. Exposures ranged from 1 min to 16 min, depending on the intensity of bioluminescence (Fig. 1B). All photographs were scanned into a computer using a Datavision 261 densitometric video camera, and digitized using Java Video Analysis software (Jandel Scientific, Corte Madera, Calif.).

Preparative image processing

Images were processed within the Khoros Image Processing Environment (University of New Mexico © 1990, 1991) or the Whiteboard graphics program (Hewlett-Packard Corp., Mountainview, Calif.). Each image was subjected to a preparative process that first adjusted companion images generated from the same root box to the same size. Resizing was performed using an "image warping routine" that aligned pixels within the root image with those of the image of the bioluminescent bacterium using bolts and corresponding bolt-holes as spatial reference points. Following resizing, all images were subjected to manual editing to remove obvious background artifacts not of plant root or bacterial origin. To maximize contrast in images, all pixel values falling within threshold levels were converted to 255 (white) and 0 (black), for root and soil, respectively (Fig. 1C) on a gray scale that ranged from 0 to 255. Similar adjustments were made for bioluminescent bacteria (255) and soil (0) in the companion image (Fig. 1D).

Image analysis of root systems and bacterial colonization patterns

Total surface areas of roots, and of bioluminescent bacteria within images, were determined by first calculating the total pixels of gray-scale value 255 in root and bioluminescent images using a histogram routine. This value was divided by the number of pixels per square centimeter to give the number of square centimeters of root or bioluminescent bacteria. The number of pixels per centimeter in each image was determined from the 1-cm-long piece of luminescent tape placed on the press plate surface. It should be noted that the data represents square centimeters of detectable bioluminescent bacteria.

Roots and bacterial-colonized areas were analyzed along a vertical axis using the seed as a reference point, and along a horizontal axis using the tap root as reference. To determine vertical distribution of roots and bioluminescent bacteria on roots, areas within images corresponding to 1-cm increments down the root system, beginning at the position of the seed, were extracted. Pixels representing root or bioluminescent bacteria were counted in the extracted subimages and surface areas calculated as described above. Horizontal distribution of bacteria away from the tap root was determined in a similar manner. In contrast to the determination of vertical distributions, however, horizontal distribution required establishment of exact reference points away from the tap root prior to extraction of subimages. This was achieved by expanding the periphery of the taproot specific distances (0.5 cm, 1.0 cm, and 2.0 cm) using a "region-growing routine," and then subtracting the taproot or expanded taproot images from appropriate press plate images representing bacterial colonization of the corresponding root system.

Visualization of E6(pUCD607) in cucumber rhizosphere

Root surface colonized by E6(pUCD607) was differentiated from rhizosphere surface area by first converting pixel values representing bacterial bioluminescence to a gray-scale value of 200. Root and bacterial images were then combined. New pixel values were assigned based on the absolute values of differences between pixels of combined images, providing differences in gray-scale values for soil alone, bacteria in soil, bacteria on roots, and roots alone. Therefore, gray-scale values for areas of root in soil = 255 (|255–0|); bacteria in soil = 200 (|200–0|); soil alone = 0

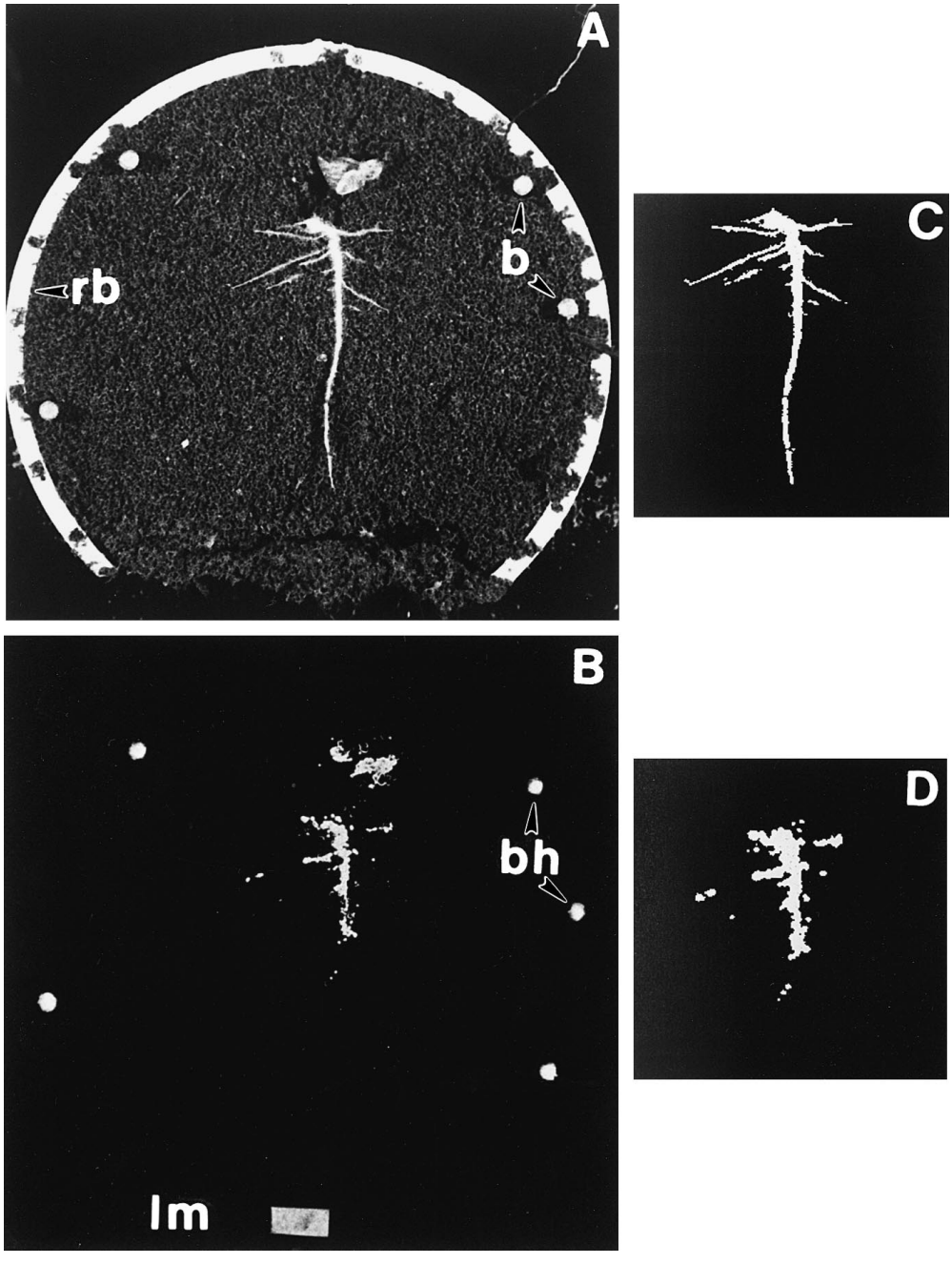

Fig. 1A–D Preparative image processing of cucumber root system and *E. cloacae* E6(pUCD607) in cucumber rhizosphere. **A** Disassembled rootbox and cucumber root system. **B** Imprint of cucumber root system on LB agar with bioluminescent E6(pUCD607) colonies. **C** Edited image of cucumber root system. **D** Edited image of E6(pUCD607). *b* bolts, *bh* bolt-holes, *lm* luminescent 1-cm marker, *rb* root box

(|0–0|); bacteria on roots = 55 (|200–255|) (Fig. 2). Total surface areas were then calculated using the histogram routine.

Lower limit of detection of E6(pUCD607) in soil and rhizosphere

Serial dilutions of E6(pUCD607) suspensions were spread onto 1-cm root segments or applied over a 10-mm^2 area of natural Galestown gravelly loamy sand field soil in root boxes. Root samples were replicated five times, and soil samples were replicated ten times, at each dilution. Press plates of root boxes containing dilution samples were made and analyzed in the same manner as described in the previous section. The experiment was performed three times.

Experimental design and statistical analysis

For image analysis, two experiments were conducted, each experiment consisting of a single soil column with 15 replicate root boxes sown with bacterized seed, and 4 replicate root boxes sown with nonbacterized seed as controls. In both experiments, no bioluminescence was detected in any of the controls. Experiments were performed twice with at least ten replicate plants for population studies along cucumber taproots. All statistical analyses were performed in SAS (Cary, NC). Mean surface areas of roots and of bioluminescent bacteria, mean $\log_{10}$ cfu E6R6(pUCD607) cm^{-1} root, and coefficients of variation were calculated using Proc Univariate. Trends were tested for significance using the Kruskal-Wallis test. Results of individual experiments were similar unless stated otherwise.

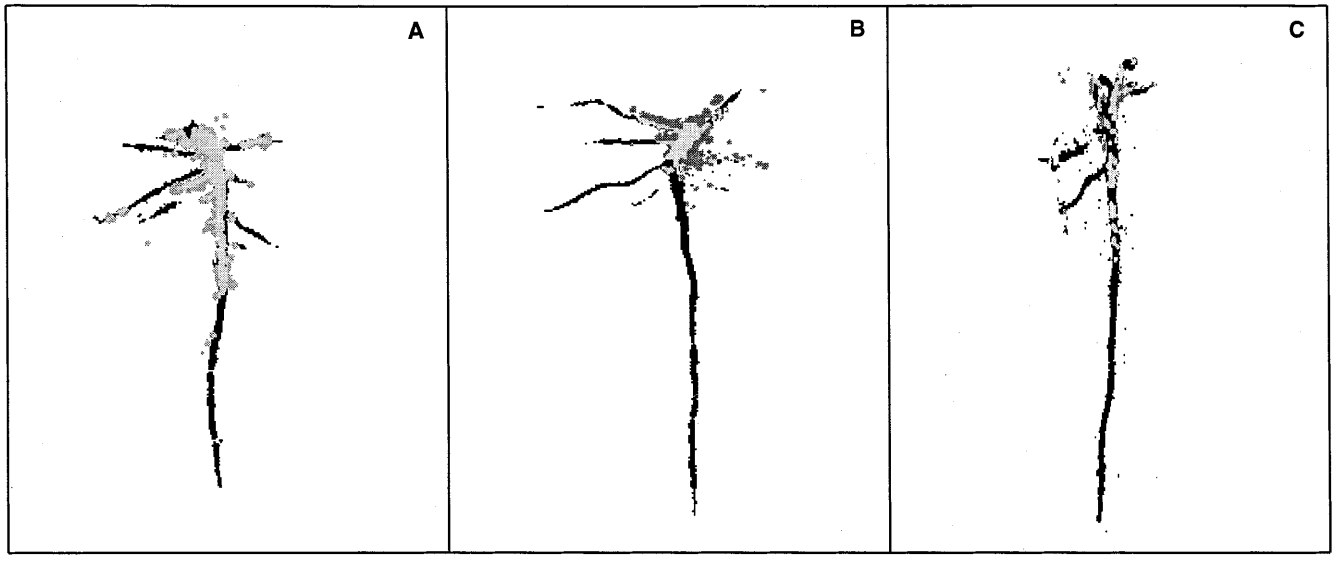

Fig. 2A–C Images from three root boxes (**A–C**) representing differences in the spatial distribution of *E. cloacae* E6(pUCD607) relative to the cucumber root system between replicate root boxes. Soil shows *white*, root *black*, E6(pUCD607) on root *light gray*, and E6(pUCD607) in soil *dark gray*

Results

Analysis of distribution of *E. cloacae* E6R6(pUCD607) along the cucumber taproot using conventional plating methods

Mean population values of *E. cloacae* E6R6(pUCD607) within the first centimeter of root were greater than log 5.5, but rapidly decreased two orders of magnitude within the second centimeter of root (Table 1). This rapid reduction in mean population values for bacteria down the root system coincided with a reduction in percentage of segments with detectable bacteria in each segment zone. As the percentage of segments with detectable bacteria decreased down the root system, the variability in mean population values increased (Table 1).

Detection limits of *E. cloacae* E6(pUCD607)

Stability of plasmid pUCD607 in *E. cloacae* E6 was determined by comparing the number of colonies in the rhizosphere resistant to Rif and Km (representing E6 populations that had maintained the plasmid pUCD607) with the number of Rif-resistant colonies (representing total populations of E6). In two of three experiments, nearly 100% of strain E6 recovered from the rhizosphere over a 72-h period maintained Km resistance. The lower limit of detection of *E. cloacae* E6(pUCD607), using bioluminescent light production on agar press plates incubated over a 16-h period, was estimated at 70 ± 30 cfu/cm root, and 1200 ± 850 cfu/10 mm^2 soil.

Image analysis of cucumber rhizosphere

Among 15 replicate plants in each of two separate experiments, root length of 3-day-old cucumber plants ranged between 3 and 7 cm. Root surface area decreased as distance increased vertically down from the seed and horizontally away from the tap root, as determined by

Table 1 Distribution of *Enterobacter cloacae* E6R6(pUCD607) populations colonizing cucumber taproots, as determined by dilution-plating (*CV* coefficient of variation)

Root segment zone[a]	Experiment 1				Experiment 2			
	Number of segments[b]	Segments colonized (%)[c]	Log cfu/root segment[d]	CV	Number of segments	Segments colonized (%)	Log cfu/root segment	CV
0–1 cm	10	100	5.51	16.3	10	100	5.56	7.9
1–2 cm	10	80	3.14	56.4	10	70	2.95	73.5
2–3 cm	7	71	2.70	70.0	10	30	1.24	165.0
3–4 cm	3	0	–	–	7	14	0.61	264.0
4–5 cm	1	0	–	–	1	0	–	–

[a] Zone is based on distance of root segment relative to position of seed
[b] Number of segments occurring in the root zone among the ten replicate plants
[c] Percentage of the root segments occurring within the zone with detectable populations of *E. cloacae* E6R6(pUCD607)
[d] Mean $\log_{10}$ value of ten replicated plants

analysis of root images generated from these plants (Tables 2, 3).

Analysis of the vertical distribution of E6(pUCD607) on cucumber roots suggested that colonization was loosely proportional to root surface area. Based on analysis of press plates, greater than 70% of the total area colonized by *E. cloacae* E6(pUCD607) occurred in the 0–1 cm zone, the region where the majority of root surface area was located. Close to 90% of the total surface area colonized by E6(pUCD607) was within the first 2 cm down the root system in experiment 1 (Table 2) and experiment 2 (data not shown). At extreme distal regions of the root system where root surface area was minimal, surface area colonized by E6(pUCD607) was detected at low percentages relative to the total area colonized, or was not detected at all (Table 2).

Horizontal analysis of colonization patterns indicated that close to 90% of E6(pUCD607) detectable with this method occurred within 0.5 cm of the tap root in experiment 1 (Table 3). E6(pUCD607) was detected as far as 2–3 cm away from the tap root, but this comprised an almost negligible percentage of total colonized area. Total area colonized by E6(pUCD607) that did not directly overlie root area, as determined by merged images of roots and bacterial colonization images (Fig. 2), was 36% in experiment 1 and 50% in experiment 2. Areas colonized by E6(pUCD607) decreased as distance increased vertically down the taproot or horizontally away from the taproot (Tables 2 and 3). This trend was highly significant in both experiments ($P \leq 0.00001$ for vertical analysis; $P \leq 0.0001$ for horizontal analysis). Analyzed images of bacterial colonization relied on press plate methodology involving bacterial growth. It is possible that bacterial growth on press plates introduced an error factor that overestimated actual colonization areas. Therefore, interpretations of data derived using this technique should be confined to comparisons of spatial distributions of bioluminescent bacteria from different regions of the rhizosphere.

Image analysis indicated that E6(pUCD607) populations did not colonize the root system in a consistent manner both on and around roots. Figure 2 represents a range of colonization patterns observed between combined images of the root system with bacterial colonization from replicate root boxes in experiment 1. Each image demonstrates that areas most highly colonized were located near the seed. However, variations in patterns ranged between individual plants. For example, areas colonized by E6(pUCD607) not directly on the root were obviously greater in some images (Fig. 2B) compared to others (Fig. 2A, 2C). In addition, inconsistent colonization by E6(pUCD607) was observed in

Table 2 Vertical analysis of total root surface area and total area colonized by *E. cloacae* E6(pUCD607) in the cucumber rhizosphere using imaging[a] (*CV* coefficient of variation)

Depth (cm)[b]	Root area				Area colonized by E6(pUCD607)			
	No. of root segments	Mean area (cm^2)	CV	% Total surface area[c]	No. of root segments colonized (%)	Total area colonized (cm^2)	CV	% Total surface area colonized[c]
0–1	15	0.61	39.5	49.3	93	0.42	33.4	71.7
1–2	15	0.25	47.7	20.0	100	0.11	100.0	18.0
2–3	15	0.12	34.2	9.7	93	0.04	180.0	6.8
3–4	14	0.10	29.4	8.1	53	0.02	255.2	3.3
4–5	12	0.08	38.7	6.5	29	< 0.01	300	< 1.6
5–6	8	0.04	66.3	3.2	0	0	–	–
6–7	1	0.04	–	3.2	0	0	–	–

[a] Data are from experiment 1. The results of experiment 2 were similar
[b] Depth is measured as distance from the seed
[c] Percentage of total area within this region

Table 3 Horizontal analysis of total root surface area and total area colonized by *E. cloacae* E6(pUCD607) in the cucumber rhizosphere using imaging[a] (*CV* coefficient of variation)

Distance from taproot (cm)	Root Area			Area colonized by E6(pUCD607)		
	Mean area (cm^2)	CV	% Total surface area[b]	Mean area colonized (cm^2)	CV	% Total surface area colonized[b]
0	0.65	24.1	52.8	0.26	59.7	42.6
0–0.5	0.31	41.9	25.2	0.28	52.4	45.9
0.5–1.0	0.16	57.9	13.0	0.05	89.8	8.2
1.0–2.0	0.09	96.5	7.3	0.02	143.7	3.2
2.0–3.0	0.02	46.7	1.6	< 0.01	140.0	< 1.6

[a] Data are from experiment 1; the results of experiment 2 were similar. Analysis by horizontal distances in measured zones was conducted by taproot expansion using a region-growing routine within the Khoros program
[b] Percentage of total area within this region

even the most densely populated regions of the root system (Fig. 2C). Although general trends indicated that E6(pUCD607) proportionally decreased horizontally away from the tap root, and vertically down the root system away from the seed, "pockets" of dense colonization could be occasionally detected in regions associated with lateral root tips, and away from the root system (Fig. 2A).

Discussion

Conventional dilution-plating methods for analysis of microbial populations on plant roots are labor-intensive and do not provide a representative visual depiction of spatial colonization patterns relative to the host plant root (Stanghellini and Rasmussen 1989). These studies typically have been limited to a description of a given bacterial strain as populations per entire root system (Loper et al. 1984; Loper and Schroth 1985), or as populations per root segment on root segments from specific regions of the root system (e.g., Bull et al. 1991). Other studies using genetic tagging with readily detectable phenotypes (Kloepper and Beauchamp 1992) have not provided replicated, representative descriptions of spatial patterns of bacteria in the rhizosphere. The image analysis method reported here provides a means to rapidly visualize the regions of roots heavily colonized by specific bacteria. This technique also allows the combination of spatial patterns from replicates within experiments, providing a representative depiction of spatial colonization patterns of specific bacteria relative to plant root systems.

Although the method described here does not provide quantitative estimates of bacterial populations on roots, the results clearly depict regions of root systems that are colonized by the bacterium of interest and are consistent with findings obtained with the conventional dilution-plating methods. Image analysis depicting bacteria on roots has been previously described (Inbar and Chet 1991) effectively demonstrating the distribution of chitinolytic activity throughout the root system. Our method differs from that previously described, and expands on the utility of image analysis for describing rhizobacteria colonization behavior. Our method does not rely on enzymatic activity detected by growth on the appropriate substrate. Instead, it utilizes bioluminescence, which enhances the contrast in images of colonies on plates. Bioluminescence can be readily transferred to most microbial systems, and if sufficient contrast is detected between colonies and agar press plates, it is not required at all (D.P. Roberts, unpublished).

Image analysis indicated that *E. cloacae* E6(pUCD607), when applied as a seed treatment, did not effectively or consistently colonize the entire root system of cucumber seedlings. Patterns of colonization were inconsistent in even the most densely populated regions of the roots within a single plant root system, as well as between plant root systems. More importantly, effective colonization was conspicuously absent from regions vertically distal from the seed and horizontally distal from the tap root. This is true for most seed-applied beneficial bacteria and is thought to be due to a number of factors including competition with the indigenous soil microflora (Bahme and Schroth 1987; Handelsman and Staab 1996).

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